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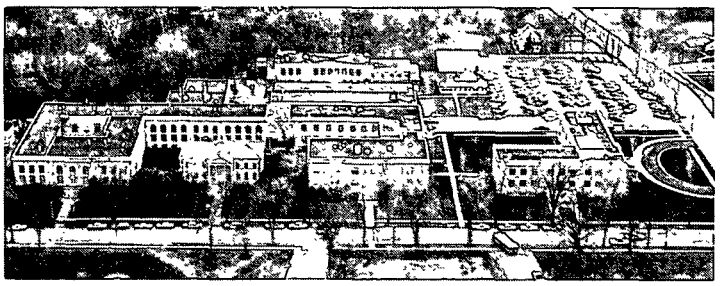

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STUDY OF THE REGENERATING CELL WALLS OF DOUGLAS-FIR PROTOPLASTS. I. PROLINE AND GLUCOSE METABOLISM

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Study of the Regenerating Cell Walls of Douglas-Fir Protoplasts. I. Proline and Glucose Metabolism

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Abstract. Radioactive proline and glucose were fed to Douglas-fir protoplasts and then recovered from the regenerating material at the plasmalemma surface. Differences in the time of initiation of radioisotope incorporation demonstrated that protein was deposited prior to polysaccharide synthesis in the wall. An increase in protein and carbohydrate content was found over the six-day period studied and the protoplasts were not observed to divide. Glucose was the dominant neutral sugar deposited although significant amounts of arabinose, galactose, and mannose were also found. Analysis of the regenerated cell wall by X-ray diffraction, carbohydrate acetolysis, histochemical staining, and microscopy suggested the absence of cellulose and the probable presence of pectin and callose, a β-(1,3)-glucan. The callose and protein deposition in the wall are both thought to be part of an overall response to cellular stress during and after protoplast formation.

Introduction

Since E. C. Cocking's (1960) isolation of higher plant protoplasts with fungal enzyme preparations, the botanical literature has been avidly concerned with the nature of protoplasts and the regenerated cell wall. Several reviews on protoplasts and cell wall regeneration are available (Willison, 1976; Preston, 1974; Cocking, 1972; Ruesink, 1971).

Until the early seventies predominantly light and electron micrographs of cell cross sections were used to elucidate gross aspects of cell wall regeneration. In 1972, Willison and Cocking used high resolution freeze-etching technology to describe microfibril synthesis on the protoplast membrane. This

approach provided workers with the opportunity to use protoplasts as tools by which biogenesis of the cell wall might be understood. The tacit hope was that protoplast wall regeneration could be used as a model of in vivo cell wall synthesis. Most of the freeze-etch studies have, in fact, revealed that fibrils are deposited on the plasmalemma. In many cases a lag was observed between enzyme removal and the initiation of cell wall regeneration. Lags of 24 hr (Willison and Cocking, 1975) to 72 hr (Robenek and Peveling, 1972) were commonly observed although wall production was also reported to begin immediately (Klein and Delmer, 1979). The general assumption underlying this approach is that the fibrils observed are cellulose and that the microfibrilar structure reflects native cellulose, i.e. cellulose I.

Biochemical studies on the metabolism of the regenerating cell wall were nonexistent until recent years. Efforts were usually frustrated by the obvious difficulties of amassing sufficient cultured protoplasts and regenerated cell wall material for analysis.

Chemical evaluations of the regenerated cell wall have proved helpful in better appreciating the protoplasts' response to wall removal. Takeuchi and Komamine (1978a) report that the dominant neutral sugar in the regenerated wall from Vinca rosea protoplasts is 1,3-linked glucose. The disaccharide, laminaribiose, is the repeating unit of callose which is generally considered to be a wound-induced polysaccharide. Herth and Meyer (1977) observed fibrillar deposition on the plasma membranes of tobacco protoplasts while X-ray analysis failed to detect significant levels of crystallinity. Upon partial acid hydrolysis or 20% alkali extraction, they respectively identified a minor product, cellobiose, and a weak cellulose II response. These facts suggest that cellulose is not as dominant as the freeze-etch studies would imply.

The disparity in these results may be resolved partially in differences arising from diversity in species, tissue source and history, and culture conditions (Hanke and Northcote, 1974). However, a potential source of confusion relates to identifying cell wall material as cellulose on purely its solubility characteristics (Brett, 1978; Takeuchi and Komamine, 1978b; Asamizu et al., 1977; Burgess and Flemming, 1974; Talmadge, et al., 1973; Updegraff, 1969). Losses due to cellulose degradation, irreversible adsorption of hemicelluloses on polysaccharides (Yllner and Enstron, 1956), and partial insolubility of some β-(1,3)-glucans (Flowers et al., 1968; Robinson, 1979) all could lead to erroneous conclusions regarding the cellulose content of cell walls. Only crystallography, partial hydrolysis (Elbein and Forsee, 1973), and/or acetolysis analysis (Green, 1963) can provide positive confirmation of the cellulosic content.

The aim of the present study was to characterize the regeneration of cell walls on Douglas-fir protoplasts from cell suspension cultures. The approach involved the analysis of various chemical and physical parameters. In addition, the ^{14}C-isotopes of L-proline and D-glucose were used, respectively, to evaluate the relative contributions of the protein and polysaccharide components in the regenerating cell wall. The protoplasts were observed to generate aberrant wall structures in response to cellular wounding.

Materials and Methods

Protoplast Culture

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] suspension cultures were derived from both needle and hypocotyl explants from greenhouse stock. Needles and hypocotyls were excised from 1 year old and 1 month old seedlings, respectively, and placed on solid agar medium. Subculturing was performed monthly

and, after 5 to 8 months, the callus clumps were gently broken apart and transferred to 150 mL of the normal suspension medium consisting essentially of half strength Murashige-Skoog (M-S)(1962). The medium was supplemented with 1.04 μM NAA while glycine, casein hydrolyzate, IAA and kinetin were deleted. The cell suspensions were cultured under constant illumination at 22°C in slowly rotating nipple flasks with a monthly addition of 100 mL of medium. After 3 months of suspension culture, cells were considered in the stationary phase and used for protoplast generation.

One day prior to enzyme treatment to remove cell walls, the suspension cultures were transferred to a protoplast medium buffered at pH 5.8. The medium consisted of mannitol (0.537M), M-S medium, citric acid — phosphate buffer (24.5 mM), ferrous ammonium sulfate (1 mM), and L-ascorbic acid (1 mM). Cellulysin and Macerase (Calbiochem, San Diego, CA) were dissolved in the protoplast medium and filter-sterilized, each at a final concentration of 2% (w/v). The enzyme mixture was added to the suspension cultures which were then incubated at 22°C on a rotary shaker (120 rpm) in the dark. After 10 h protoplasts were ubiquitous. They were washed with the protoplast medium 3 times and collected by centrifugation at less than 75 x g.

Cell Wall Regeneration

The protoplasts were split into 2 equal volumes and either L-(U-^{14}C) proline or D-(U-^{14}C) glucose (ICN Pharmaceuticals, Inc., Irvine, CA) was introduced with respective specific radioactivities of 250 and 240 mCi/mmole. Each radioactive species was applied at a level of 0.1 μCi/mL of medium. Aliquots (5.0 mL) of protoplast suspension were aseptically transferred to test tubes with metal closures and rotated (1 rpm) under constant illumination (1.6, 1.2, and 0 μW/cm^2 at peak wavelengths of 455, 640, and 735 nm, respectively). Samples were

evaluated at cell wall regeneration times of 0, 1/2, 1, 2, 3, 4, 5, and 6 days. Each sample time reported in the isotope experiments represented an average of 4 replications.

Parallel controls were conducted during the wall regeneration studies to check that radioactivity uptake by the walls was both an enzyme-mediated and cytologically organized event. The first control was to establish whether physical sorption processes played a significant role in final cell wall radioactivity. After radioisotope was added to the protoplasts as above, cells were immediately placed in a boiling water bath for 10 min to inhibit enzyme activity. The second control was to establish the role of protoplast integrity in the uptake of radioactivity. After radioisotope addition, the protoplasts were ball-milled and reduced to debris. In both controls the cell suspensions were returned to the rotary device and treated as above with the exception that only duplicate samples were evaluated.

Cell Wall Isolation

Regenerated cell walls were separated from cytoplasmic materials by using a modification of Lamport's method (1965). Cells were washed with 1M NaCl and homogenized for 5 min in a veined Virtis mixer jar using 3 mm glass beads. The washing and centrifugation schedule is outlined in Table 1. Clean cell walls were evaluated for activity in a scintillation counter (Smeltzer and Johnson, 1977) and counted to a ±2% statistical error. Walls were retrieved from the dioxane-based cocktail by two washes with acetone followed by one with water.

Analytical Techniques

Several methods were employed to characterize the chemical constitution of the regenerated cell wall. In evaluating the carbohydrate fraction, alditol-acetates

of hydrolyzed cell wall polysaccharides were made according to the method of Borchardt and Piper (1970). A Packard model 417 gas-liquid chromatograph (GLC) equipped with a flame ionization detector and a data system was used.

The crystallinity of the regenerated cell was determined by X-ray diffraction (Atalla and Whitmore, 1978). Samples were pelletized, scanned, and compared with the spectra of β-(1,4)-glucan(cellulose) and β-(1,3)-glucan-(bacterial callose). The β-(1,4)-linked polymer is the most abundant constituent in normal cell walls while the β-(1,3)-glucan is commonly synthesized in response to wounding (Clarke and Stone, 1963).

Regenerated wall material and filter paper (control) were separately evaluated for cellulosic content by the acetolysis procedure of Braun (1943). Detection of octaacetyl-α-cellobiose using this method is a positive indication of the presence of cellulose. The acetolysis products were evaluated by both thin-layer chromatography (TLC) on silica gel plates and by GLC on an OV-17 column using octaacetyl-α-cellobiose and glucose pentaacetate as standards.

Cell walls regenerated on protoplasts were evaluated for amino acid composition. Samples were hydrolyzed in 1 mL of constant-boiling HCl and 0.5 μL of mercaptoethanol for 22 h at 110 ± 0.5°C. Humin formed was removed by mild centrifugation and the supernatant dried over NaOH pellets *in vacuo* at 60°C (Brenner *et al.*, 1965). Amino acid composition was determined on a Beckman model 119CL amino acid analyzer equipped with a Beckman model-126 computer.

Microscopy

Hypocotyl-derived cell populations from the original suspension cultures, isolated protoplasts, and cells with regenerated walls were separately fixed in 2% glutaraldehyde plus 2% acrolein in protoplast medium for 3 h at room temp and some

samples were post-fixed in 1% OsO_4. Of those treated with osmium, some were dehydrated in an alcohol series, plated on a glass-supported polylysine film (Tsutsui et al., 1976), dried over argon gas in vacuo, and viewed in a JEOL JSM-U3 scanning electron microscope (SEM) at 20 kV. Others were dehydrated in an acetone series and embedded in Spurr resin (E. F. Fullam, Inc., Schenectady, N. Y.). Both 10 and 0.1 μm sections were made. The thicker sections were used in light microscopy for the detection of lignin and callose in the cell wall region using a Zeiss photomicroscope equipped with a UV source. Lignin was detected by autofluorescence and callose by a specific reaction with a 0.01% solution of aniline blue (Eschrich and Currier, 1964; Currier and Strugger, 1956).

The ultra-thin sections were post-stained with uranyl acetate (5% in 50% ethanol) and lead citrate (0.5% in water). Samples were examined on an R.C.A. model EMU-3F transmission electron microscope (TEM).

Samples not osmium-treated were reacted separately with several electron-dense stains. The hydroxylamine-iron staining scheme (McCready and Reeve, 1955; Gee et al., 1959) was employed to detect the presence of pectin in the wall. As a control of staining reactivity other samples were similarly treated but boiled in 0.5% aqueous ammonium oxalate for 24 h prior to staining. The second staining sequence evaluated the availability of (1,4)-sugar linkages in the wall. The reaction involved periodic acid oxidation of adjacent sugar hydroxyls followed by reaction with thiosemicarbazide and osmium reduction (Seligman et al., 1965). Both staining sequences were followed by osmium fixation, embedding, and ultrathin-sectioning (0.1 μm) as before.

Results

Radioactivity from ^{14}C-Proline and ^{14}C-Glucose in the Regenerating Cell Wall

The biosynthesis of protein and polysaccharide material in the regenerating cell walls of Douglas-fir needle-derived protoplasts was demonstrated by the respective uptake of radioactive L-proline and D-glucose into polymeric forms as shown in Figure 1. Proline was rapidly incorporated into the wall fraction while a lag was observed in the uptake of glucose.

The hypocotyl-derived protoplasts also incorporated both proline and glucose into the regenerating wall as shown in Figure 2. On a similar dry weight basis with the protoplasts in Figure 1, the incorporation of radioactivity was observed to be less for the hypocotyl cells. However, a similar lag was observed in both cultures between the onset of proline incorporation and the beginning of glucose uptake.

The remainder of the results, with the exception of the microscopic study, pertain to experiments involving the needle-derived tissue only.

Minor binding of radioactivity from both precursors was detected in the boiled controls and this amount was subtracted from the activity reported in Figures 1 and 2. The second control, consisting of cytoplasmic and wall debris, indicated that only organized cells were involved in the uptake of radioactivity.

Chemical and Physical Analysis of the Regenerated Cell Wall

Wall regeneration for Douglas-fir needle-derived protoplasts appeared to begin at about 30 h after enzyme removal as indicated by the intercept in Figure 3. From that time onward the wall exhibited a constant weight increase throughout the period studied.

Total carbohydrate analysis of the regenerated cell wall is displayed in Table 2. Glucose is the dominant neutral sugar followed by mannose, galactose, and arabinose. Only minor amounts of xylose, ribose, fucose, and rhamnose were detected. The percent increase of the individual sugars in the wall is plotted in Figure 4. The only polysaccharide material actively synthesized at the initiation of wall regeneration (ca. 30 h) is a polymer containing mannose. The other major carbohydrates are found only after 2 days.

The results from the amino acid analysis of the wall regeneration period studied are displayed in Table 3. The general trend of amino acid composition is illustrated in Figure 5. A more detailed characterization of the protein component found in the regenerated cell wall is reported in part 2 of this investigation (Robinson and Johnson, 1980).

The results from the X-ray diffraction analysis are plotted in Figure 6. Cell wall material recovered from various stages of wall regeneration is shown along with walls of suspension cells. Native cellulose and bacterial callose (Harada, 1977) are standards. The regenerated and suspension cell walls were not sufficiently crystalline to demonstrate the presence of ordered cellulose. Even after high temperature annealing in glycerol, no increase in order was detected.

Acetolyzates were made of the X-ray analyzed regenerated wall samples reported above to test chemically for the presence of cellulose. Only the control yielded the primary cellulose hydrolysis product, octaacetyl-α-cellobiose, thereby confirming the absence of β-(1,4)-glucan in the regenerated cell wall.

Structural and Histochemical Study

Light and electron microscopy provided insight into the cell wall regeneration process. Shown in Figure 7 is the cross section (7a) and cell surface (7b) of Douglas-fir protoplasts (i.e., wall regeneration time = 0 days) which exhibited little evidence of a cell wall. Total removal of the cell walls required a 10 h incubation period with the fungal enzymes. This situation principally resulted from the large quantities and high concentrations of suspension cells required to conduct an investigation of this scope. Even under these extended conditions, minor amounts of wall residue existed on the otherwise clean cell membranes of some protoplasts (see Fig. 7b).

Since the protoplasts did not exhibit a measurable cell wall (Fig. 7a), the remainder of the microscopy study involved detecting qualitative similarities and differences which existed between the suspension cell walls and the regenerated cell walls. After 6 days of protoplast culture for wall regeneration, bubble-like structures were observed on the cell surface of some protoplasts as shown in Figure 8. The balloon-like materials on the cell surface suggested callose formation (Fahn, 1974) and a positive reaction to aniline blue in the wall region of cells from the same population was detected. A summary of these and other results are displayed in Table 4.

Discussion

Douglas-fir protoplast cultures were incubated with radioactive L-proline and D-glucose in order to observe their relative metabolic involvement in the regenerating cell wall. Reproducibly characteristic was the initiation of proline incorporation prior to glucose uptake (see Figs. 1 and 2). While lags in microfibril synthesis have been reported (Burgess and Linstead, 1979; Grout,

1975; Marchant and Hines, 1972), this is the first study which demonstrated a potential relationship between protein and carbohydrate components in the regenerating cell walls of higher plants.

The rate of wall protein and polysaccharide anabolism appears to vary in Douglas-fir cultures from the needle-derived to hypocotyl-derived tissues. It is unclear, however, whether this response is in any way related to some fundamental and characteristic differences between the two cell types or is just indicative of variation in the lag associated with wall regeneration. Observed differences might, in fact, disappear if a statistical study were undertaken.

Cell wall weight increased linearly over the period of synthesis studied (see Fig. 3). As expected, wall weight increase is simultaneous with ^{14}C-glucose uptake, clearly suggesting that glucose incorporation is indicative of cell wall regeneration. In addition, a constant weight increase may suggest that no new carbohydrate synthesis sites formed (i.e., no cells divided) over the period studied. While differences in cell density were not monitored, no active cell division was observed for up to 12 days of protoplast culture. Other workers have drawn similar conclusions although whether cell division occurs appears to be culture-dependent. Kirby (1980) and Strmen (1979) observed that the gymnosperm protoplasts derived from suspension cultures of Douglas-fir (needle) and Norway spruce, respectively, failed to divide after enzymatic treatment. In contrast, protoplasts from suspension cultures of carrots (Asamizu and Nishi, 1980) and Vinca rosea (Takeuchi and Komamine, 1978a) all divided within several days of culture. Within cultures, cell division was reported to be dependent on illumination intensity (Shepard and Totten, 1977) and medium molarity (Pearch and Cocking, 1973). Callusing (Kirby and

Cheng, 1979) and plant regeneration (Gamborg and Shyluk, 1976) is more commonly reported from protoplasts derived from intact tissues.

Special culture conditions are clearly necessary when generating large populations of protoplasts. The concentrations of wall degrading enzymes, time, and incubation temperature are usually in excess of standard protoplast methods. For example, in order to conduct a large scale study of the molecular weight distribution of cellulose from the regenerated cell wall, Asamizu et al., (1977) reported that 5% cellulase and 1% pectinase were used to complete wall removal in carrot cultures. Takeuchi and Komamine (1978a), in order to enzymatically remove and then chemically characterize the regenerated wall of Vinca rosea cell suspensions, required 2% cellulase and 1% pectinase. The cultures in those studies were maintained at 30°C for 6 and 5 h, respectively. In spite of comparable conditions used in this study, data in Tables 2 and 3 demonstrate that a chemically measurable cell wall remains on the protoplasts. No residual wall, however, could be detected on ultrathin sections in the TEM (see Horine and Ruesink, 1972). Nevertheless, SEM analysis revealed that small portions of wall material remain in some cases despite the fact that protoplasts are generated (see Fig. 7b).

A polymer containing mannose is synthesized as the initial polysaccharide in the wall and is probably responsible for the uptake of ^{14}C-glucose prior to 2 days of protoplast culture since glucose can interconvert to mannose (Harran and Dickinson, 1978). In yeast protoplasts, mannan-protein deposition occurs in concert with the synthesis of β-(1,3)-glucan in the regenerating wall (Kopecká and Farkas, 1979). Mannan may be involved in the glycosylation and binding of the protein to the cell walls of the Douglas-fir system in an analogous fashion.

The composition of the regenerated cell wall includes glucose as its major carbohydrate with significant amounts of hemicellulosic components. Common hemicelluloses in most conifers are galactoglucomannan (0.1:1.0:3.0) and arabinogalactan (1:5) which may be similar in form to the constituents of the regenerated cell wall. If the regenerated wall hemicelluloses are similar, then a glucan will exist since only a minor amount of glucose could be involved in the hemicellulosic fraction.

Several analyses of the regenerated wall were made to define the nature of the glucan. Analysis by X-ray diffraction techniques revealed that the regrown wall failed to exhibit a crystalline pattern characteristic of cellulose; Herth and Meyer (1977) experienced similar results. Their X-ray diffractograms of tobacco regenerated walls revealed the absence of any crystallinity. In contrast, their negatively-stained cell surfaces showed massive arrays of microfibrils and severe alkali extractions induced only a weak crystalline pattern characteristic of cellulose II. In this regard Talmadge and coworkers report (1973) that primary wall microfibrils exhibit a highly crystalline core. In addition, microfibril crystallization occurs due to enzymatic induced alignment of glucose chains at the plasmalemma surface (Brown and Montezinos, 1976). Therefore, any observation that microfibrils are present in the regenerated wall would be complemented with a positive crystallographic analysis. The intact plant cell wall has cellulose as its major component. In both natural and suspension systems, cells are able to synthesize β-(1,3)- and β-(1,4)-linked glucans (Brett, 1978; Van DerWoude et al., 1974; Bauer et al., 1973; Talmadge et al., 1973; Clarke and Stone, 1963), although the former is only found naturally in either specialized or wounded plant parts.

Researchers suggest that cultured plant cells may exhibit aberrant wall structures. Chanzy and coworkers (1979) analyzed a suspension culture of rose cell walls by both electron and X-ray diffraction. Their study revealed the strong presence of cellulose IV amid the unexpected absence of cellulose I. In the regenerated wall of rose protoplasts Pearce and Cocking (1973) reported that fibrillar synthesis was observed to be dependent on medium molarity after protoplast formation.

Acetolysis of the regenerated walls of Douglas-fir protoplasts revealed the absence of cellulose. These results confirmed the lack of any fibrillar material present on the cell membrane. Therefore, no significant amounts of a β-(1,4)-glucan appear to be present. These findings are in contrast to the analysis of Douglas-fir suspension cell walls(Burke et al., 1974). By methylation analysis Burke and coworkers demonstrated that 22% of the cell wall was cellulose while the (1,3)-glucan was not detected.

In the present study other wall components were exclusively in either the regenerated or suspension cell wall of Douglas-fir. While lignin was observed in the suspension cell wall, its presence went undetected after enzyme treatment. Talmadge et al. (1973) suggest that if suspension cells model a primary wall, then they should be devoid of lignin. However, others (Fukuda, 1978; Whitmore, 1978; Barnoid, 1965) demonstrated the presence of lignin in cultured tissues of poplar, slash pine, and sequoia. The inability to detect lignin after 6 days culture may also be related to the suggestion that lignin synthesis increases with age of culture (Fukuda, 1978).

After wall-degrading enzyme removal, the Douglas-fir protoplast culture exhibited ultraviolet fluorescence upon reaction with aniline blue after 6 days

of protoplast culture. Some cell surfaces were characterized by numerous "bubble-like" structures substantially smaller than the "blebs" found on the surface of Douglas-fir suspension cells (Parham and Kaustinen, 1976). The cells with surface "bubbles" were likely those with substantial wall material (i.e., regenerated wall) which fluoresced as callose. Since callose is widely synthesized in response to wounding, it is reasonable that protoplasts have experienced a structural attack by the action of the wall-degrading enzymes and/or removal of the original cell wall. "Protoplasts are, after all, injured cells which have to overcome many obstacles on the way to becoming healed" (Galston, 1978). The cells with surface "bubbles" then were those which attempted to repel the "attack."

The suggestion that callose may be a dominant polymer in wall regeneration is not new. Vinca rosea protoplasts incorporated the majority of glucose into the β-(1,3)-linked polymer (Takeuchi and Komamine, 1978a). Protoplasts generated by plasmolysis in Elodea were induced to produce callose (Prat and Roland, 1971) while Herth and Meyer (1977) implied that the enzymatically generated protoplasts of tobacco contained callose in the regrown wall. Strmen (1979) demonstrated that Norway spruce protoplasts contained enzyme systems to synthesize either β-(1,3)- or β-(1,4)-linked glucans. Callose may possibly assemble in fibrillar form in higher plants (Marchessault, 1979) as reported in yeast (Kreger and Kopecká, 1978) during cell wall regeneration. However, other evidence suggests that the presence of callose in the regenerated wall is not universal. Horine and Ruesink (1972) found that the regenerated walls of Convolvulus were devoid of callose.

Pectin was identified in both suspension and regenerated cell walls of Douglas-fir cultures. Takeuchi and Komamine (1978a) reported pectin synthesis

and deposition in Vinca rosea protoplasts. They detected lower but yet significant yields (<10%) when compared with suspension cells. However, Hanke and Northcote (1974) reported soybean protoplast walls devoid of pectin while later (Boffey and Northcote, 1975) observing that the rate of pectin synthesis in tobacco leaf protoplasts was similar before and after plasmolysis.

The presence of protein in the regenerated wall was detected by Herth and Meyer (1977) and Willison (1976) although composition was never reported. Figure 5 illustrates some important features of the regenerating cell wall. There was a net increase in cell wall protein content over the period studied. This plot of amino acids probably indicates that deposition is a simple process only involving a few proteins. In this case the role of the protein(s) in the regenerating wall seems not to be similar to extensin (Lamport, 1978), but rather is probably associated with a wounding response.

The results in the present study provide insight into cell wall regeneration by protoplasts from suspension cells. With few exceptions (Davey and Mathias, 1979; Williamson et al., 1977) the bulk of freeze-etch studies on protoplasts have dealt with cells isolated from intact tissues. In general, such cells respond by renewed wall synthesis within 24 h of isolation and consequently may differ significantly from suspension cells since the latter are dedifferentiated through culturing. Robenek and Peveling (1977) and Pearce and coworkers (1974) report that vivid cytological differences exist between protoplasts from cultured and intact sources.

In conclusion, cell wall regeneration by Douglas-fir protoplasts originating from cell suspensions appears not to be a model system for studying wall biogenesis. Rather, wall regeneration examined here seems to be largely a response to wounding and a protein, probably also related to wounding, is

deposited prior to polysaccharide synthesis. These two aspects of wall regeneration are undoubtedly related although the manner of action is not yet understood.

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Table 1. The washing and centrifugation schedule used to separate cytoplasmic and radioactive materials from the regenerating cell walls. The cells were first ball-milled at slow speed for 5 min in 1N NaCl to separate the cell walls, then transferred to 50 mL centrifuge tubes and washed according to this schedule.

Washing Solvent	Centrifugal Force, x g	Centrifugation Time, min
1M NaCl	800	0.5
Water[a]	800	0.5
Water	800	0.5
Water	29,000	20.0
Acetone	29,000	10.0

[a]Double distilled water used throughout all experiments.

Table 2. The neutral sugars present in the regenerating cell wall. At zero regeneration time the carbohydrate analysis reveals that the residual suspension cell wall is about 13% polysaccharide. The dominant sugar in both the residual and the regenerating wall is glucose.

Sugar	Weight, % Regeneration Time, days							
	0	0.5	1	2	3	4	5	6
Glucose	6.34	4.66	4.98	6.31	16.64	21.98	21.22	27.37
Arabinose	2.88	2.31	2.04	2.46	2.98	3.34	3.11	3.44
Galactose	1.89	1.36	1.30	1.79	2.86	3.50	3.67	4.07
Mannose	0.62	0.77	0.93	1.43	3.62	4.67	5.21	5.29
Xylose	0.63	0.48	0.46	0.38	0.52	0.62	0.52	0.50
Rhamnose	0.43	0.25	0.90	0.30	0.32	0.32	0.25	0.30
Ribose	0.26	0.19	0.17	0.41	0.36	0.30	0.11	0.16
Fucose	0.09	0.03	0.39	0.07	0.09	0.08	0.07	0.08
Total	13.14	10.05	11.17	13.15	25.39	34.81	34.16	41.21

Table 3. The molar percentages of amino acids from acid hydrolyzed cell wall samples. Arranged according to average descending concentration, glycine and alanine alone account for over 25% of the protein fraction. Also, note the fairly good agreement in molar ratios as regeneration proceeds even though the net deposition of protein increases as shown in the bottom row.

Amino Acid[a]	MOLAR PERCENTAGE Regeneration Time, days 0	0.5	1	2	3	4	5	6	Average
Glycine	13.7	13.8	15.8	13.1	13.5	14.3	13.3	13.9	13.9
Alanine	11.4	10.3	11.9	12.1	13.8	14.5	12.9	14.4	12.7
Aspartic acid	10.4	11.2	9.5	9.4	10.5	10.2	9.7	9.9	10.1
Serine	9.7	9.2	9.3	10.6	9.4	9.7	8.5	7.9	9.3
Glutamic acid	7.5	8.8	9.1	7.0	6.7	7.5	8.2	8.1	7.9
Leucine	8.1	7.7	8.7	7.5	7.5	7.8	7.3	7.4	7.8
Proline	5.5	6.5	7.6	6.9	7.2	7.6	6.1	7.3	6.8
Lysine	6.0	4.6	4.1	4.6	4.6	4.4	2.4	3.9	4.3
Threonine	4.2	5.0	4.3	4.4	3.8	3.9	4.5	3.8	4.2
Valine	3.1	4.5	3.8	4.0	3.4	3.4	4.0	3.7	3.7
Phenylalanine	4.7	3.4	3.4	4.4	4.3	3.3	3.1	3.3	3.7
Arginine	4.2	3.4	3.1	3.5	3.5	3.4	3.4	3.0	3.4
Hydroxyproline	3.4	3.4	1.9	2.5	1.9	1.8	4.0	3.4	2.8
Isoleucine	2.1	2.7	2.2	3.0	2.3	2.2	2.5	2.3	2.4
Tyrosine	2.4	2.1	2.2	2.3	2.4	2.1	2.3	2.2	2.3
Histidine	1.9	1.8	1.7	1.8	1.7	1.7	1.6	1.5	1.7
Methionine	1.1	0.4	0.7	1.4	0.7	1.1	0.3	0.7	0.8
Total weight % of Cell Wall	19.3	15.8	17.4	16.8	26.2	24.8	31.4	29.3	

[a]Neither cysteine nor cystine were detected.

Table 4. A summary of the microscopy study. The suspension and regenerated cell walls are compared and observed to differ in their response to callose and lignin detection. Both types of cell walls contain pectin and (1,4)-linked polysaccharides.

Component Tested	Response: Suspension Cell Walls	Response: Regenerated Cell Walls
Cellulose microfibrils	N.D.[a]	-[b]
Callose (β-1,3-glucan)	N.D.	+[c]
Lignin	+[d]	-
1-4 Linked carbohydrates	+[e]	+
Pectin	+[f]	+

[a] Not Determined
[b] As detected by standard shadowing and freeze-fracture techniques.
[c] As detected by reaction with aniline blue.
[d] As detected by autofluorescence.
[e] As detected by oxidation with periodic acid, followed by reaction with thiosemicarbazide, and reduction of osmium tetroxide.
[f] As detected by forming hydroxamic acids and then reacting with ferric chloride.

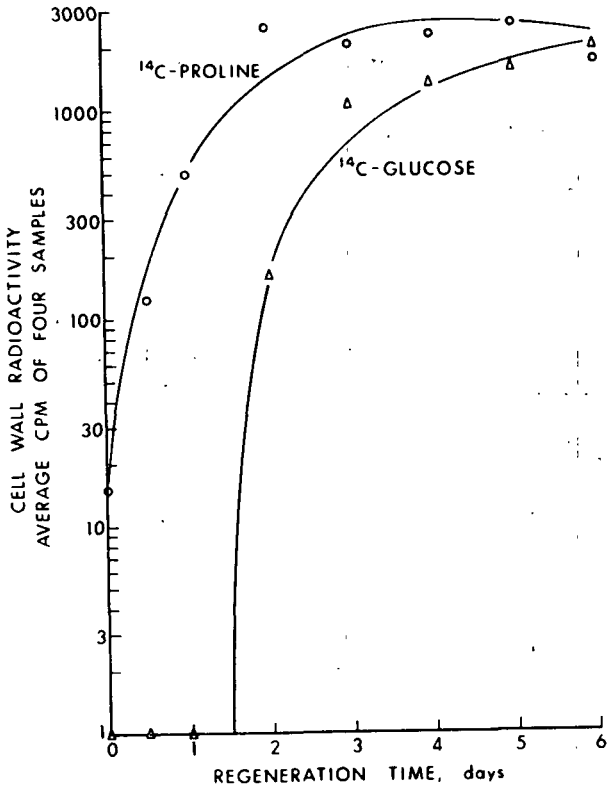

Fig 1. The recovery of radioactivity originating from labelled proline and glucose in the regenerating cell wall of Douglas-fir needle-derived protoplasts. Note the time lag which exists between the uptake of the polysaccharide and protein components. The protoplasts were maintained at constant illumination and remained green over the period studied.

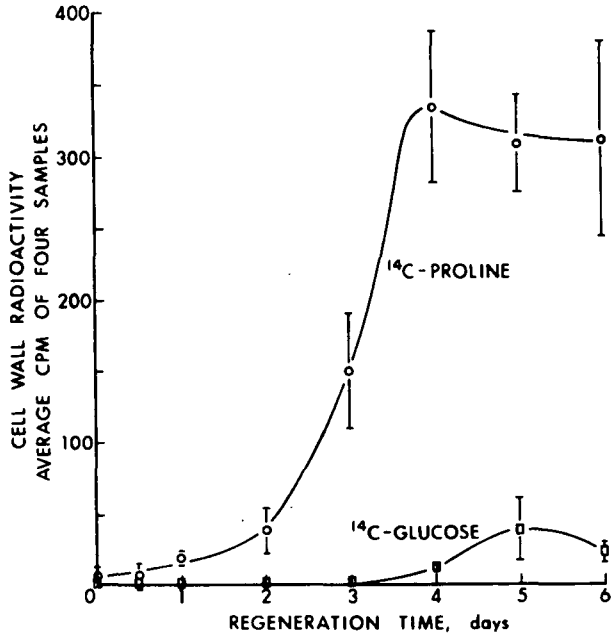

Fig. 2 The recovery of radioactivity originating from labelled proline and glucose in the regenerating cell wall of Douglas-fir hypocotyl-derived protoplasts. The plot is as described in Fig. 1 except that the protoplasts are from hypocotyl-derived tissue. A similar lag is observed in the differential uptake of proline and glucose. The protoplasts remained green but did not exhibit the levels of radioactivity found in the needle-derived protoplasts.

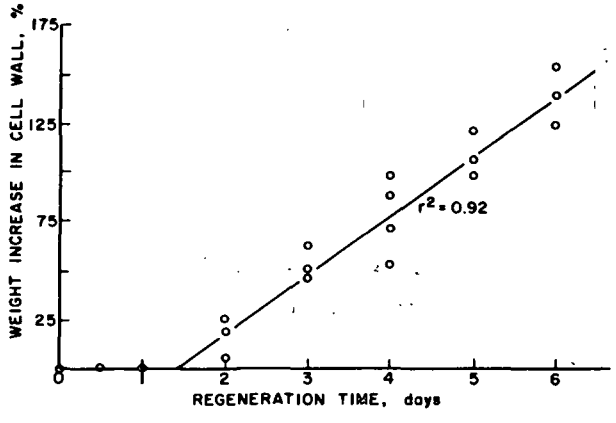

Fig. 3 Cell wall weight increase for needle-derived protoplast culture. A linear relationship exists in the deposition of cell wall material after about 30 h. The constant wall weight increase suggests that no cells divided during the period studied.

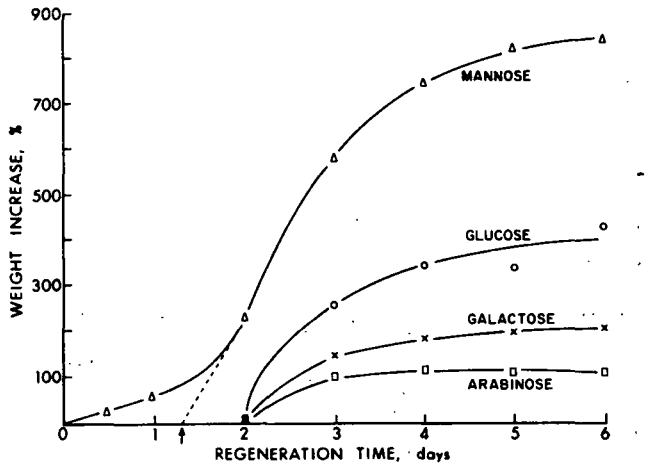

Fig. 4 The synthesis of neutral polysaccharides in the regenerating cell wall. The increase in neutral sugars beyond the time of protoplast generation shows the initial formation of a polymer containing mannose. Mannose incorporation is extrapolated to 30 h to indicate the intersect of cell wall weight increase (Fig. 3) and ^{14}C-glucose uptake (Fig. 1).

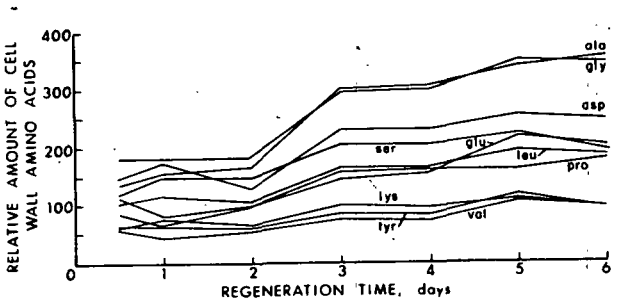

Fig. 5 Relative concentration of selected amino acids present in the acid hydrolyzates of regenerated cell wall samples. An orderly trend is observed in the relative increase in amino acids suggesting that protein is accumulated in the wall during the period studied. The amino acids not shown here are listed in Table 3 and were observed to exhibit similar trending but were omitted on this plot for clarity.

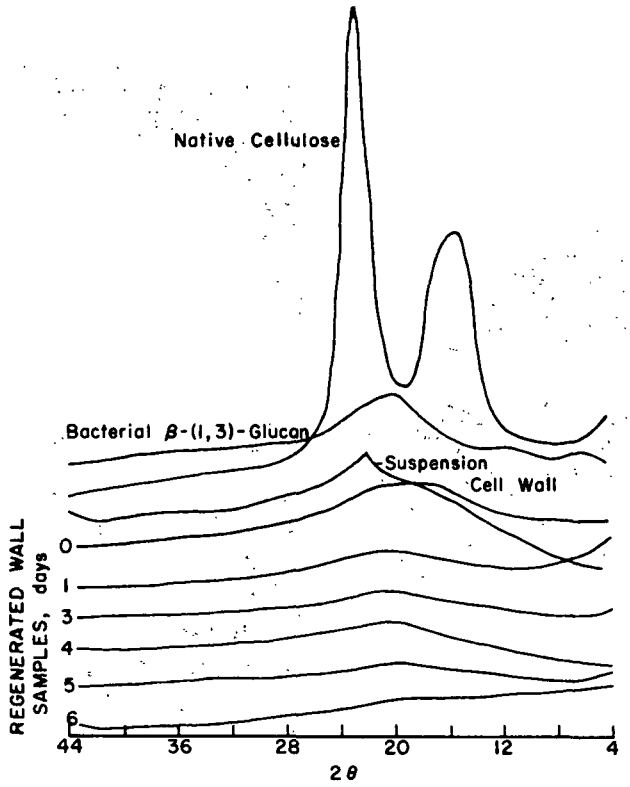

Fig. 6 X-ray diffractograms of regenerating and suspension cell walls compared with those of native cellulose and bacterial β-(1,3)-linked glucan (i.e. curdlan, Harada, 1977). The general shift of ordering by the regenerating walls is to 20°, the most ordered point of the native β-(1,3)-glucan and not to the 16° and 23° region which is characteristic of cellulose. High temperature glycerol annealing of the regenerating cell wall did not increase crystallinity or shift the diffraction pattern (not shown), confirming the absence of crystalline cellulose.

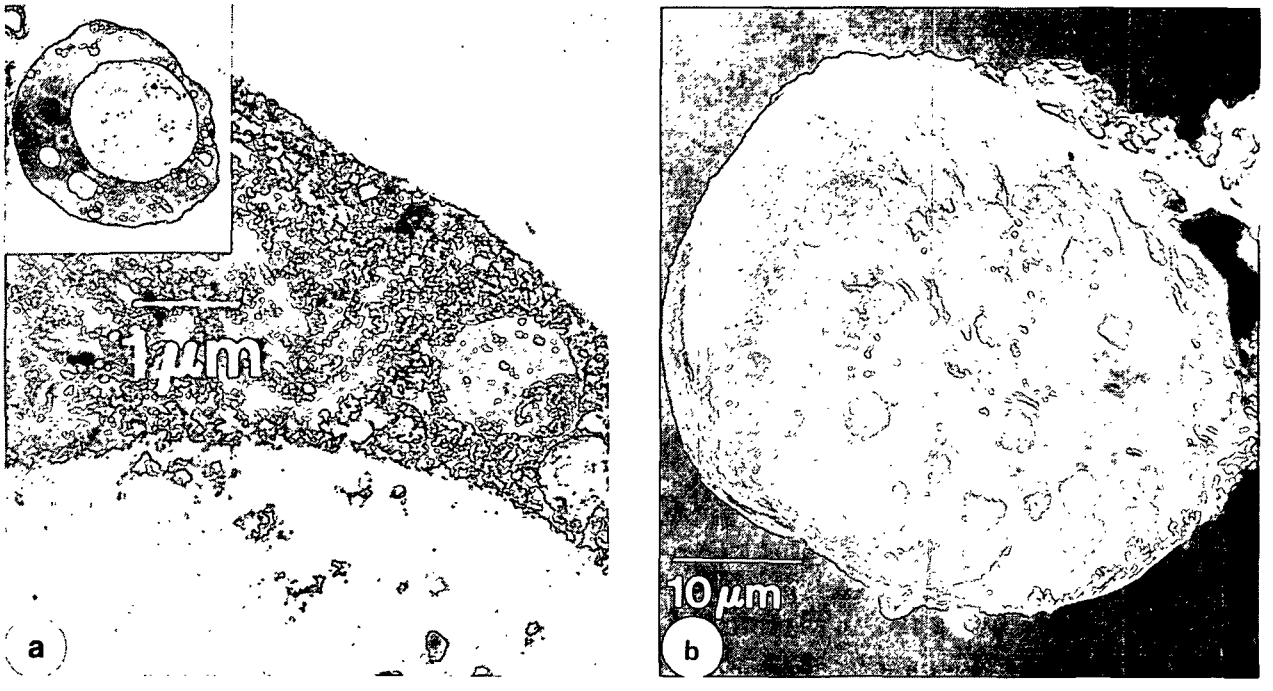

Fig. 7 A cross section (7a) and surface view (7b) of Douglas-fir protoplasts. Figure 7a shows that the cell wall has been removed (14,000X) and that the cell takes on the characteristic protoplast spherical shape (inset). However, Fig. 7b shows that some residual wall remains on the protoplast even though what may appear to be cell organelles are observed beneath the clean and skin-like membrane surface (1700X).

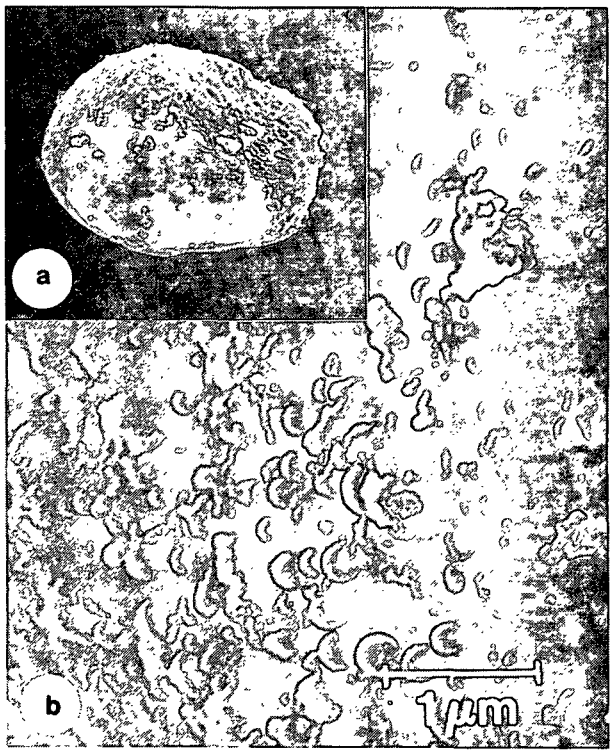

Fig. 8 a. Surface view of a cell after 6 days wall regeneration. Shown is a cell which has regenerated a cell wall. b. The wall consists of "bubble-like" material suggestive of callose (20,000X).